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Screening for antisense modulation of dystrophin pre-mRNA splicing

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Abstract

Most gene therapy approaches to genetic disorders aim to compensate loss-of-function by introducing recombinant cDNA-based minigenes into diseased tissues. The current report represents an ongoing series of studies designed to correct genetic mutations at the post-transcriptional level. This strategy modifies the binding of components of the spliceosome by high affinity hybridisation of small complementary (antisense) RNA oligonucleotides to specific pre-mRNA sequences. These, so-called 'splicomer' reagents are chemically modified to impart bio-stability, and are designed to cause skipping of mutant frame-shifting exon sequences leading to restoration of the reading frame and an internally deleted but partially functional gene product. For instance, Duchenne muscular dystrophy is generally caused by frame-shift mutations in the dystrophin gene, whereas in-frame deletions of up to 50% of the central portion of the gene cause Becker muscular dystrophy, a much milder myopathy, which in some cases can remain asymptomatic to old age. In the *mdx* mouse model of Duchenne muscular dystrophy, a mutation in exon 23 of the dystrophin gene creates a stop codon and leads to a dystrophin-deficient myopathy in striated muscle. In previous studies, we have demonstrated that forced skipping of this mutant exon by treatment of *mdx* muscle cells with splicomer oligonucleotides can generate in-frame dystrophin transcripts and restore dystrophin expression. Here, we report the results of an optimisation of splicomer sequence design by the use of both high-throughput arrays and biological screens. This has resulted in specific and, importantly, exclusive skipping of the targeted exon in greater than 60% of dystrophin mRNA, leading to the de novo synthesis and localisation of dystrophin protein in cultured *mdx* muscle cells. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Genetic mutations; Dystrophin gene; Splicomer sequence design

1. Introduction

Duchenne muscular dystrophy (DMD) is caused by mutations in the X-linked DMD gene leading to premature truncation of the large cytoskeletal protein, dystrophin [1]. However, mutations that lead to in-frame deletions of up to 50% of the central part of the dystrophin molecule cause a milder myopathy, Becker muscular dystrophy (BMD), which can in some cases remain asymptomatic to old age [2,3]. Over 92% of patients follow this 'reading frame rule'. The rationale behind recent gene therapy approaches to genetic disorders has been to compensate loss-of-function by introducing recombinant cDNA-based minigenes into diseased tissues [4–6]. Indeed, a degree of success has been achieved in muscle of the *mdx* mouse model of DMD by our laboratory and others [4,5,7–16], and Phase I clinical trials are ongoing in France (see reports from this meeting). Nevertheless, difficulties remaining include the packaging of large DNA molecules (e.g. dystrophin cDNAs) in viral vectors, immune responses, low efficiency gene transfer to muscle in vivo [17–22], so the development of alternative pharmacological gene therapeutic approaches remains vital.

Since the discovery of low numbers of dystrophin-positive (revertant) myofibres in many DMD patients and the *mdx* mouse model of the disease, the underlying mechanism by which these fibres arise, and the significance of low-level dystrophin expression in dystrophic muscle, has been the subject of much speculation [23–25]. Furthermore, molecular analysis of patients regarded as 'exceptions' to the reading frame rule (by exhibiting milder phenotypes than predicted from their genomic mutations) are of great interest, as it may be possible to mimic, using a therapeutic agent, the mechanism of dystrophin expression in their skeletal muscle. Two main theories are prevalent for explaining dystrophin expression in generally dystrophin-deficient muscle, both of which may be true. First, secondary genomic mutations in isolated nuclei may restore the reading frame of mutant DMD genes. This has been used to explain the gradual increase of dystrophin-positive fibres with age in the *mdx* mouse [26], but does not explain the absence of this phenomenon in cardiac muscle cells. Indeed, the age-related increase in such fibres may reflect the selective advantage of dystrophin-positive fibres, as a similar

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increase with age from 50–90% is also found in DMD carrier females [27]. Second, there is evidence from reverse transcriptase-polymerase chain reaction (RT-PCR) analysis that genomic mutations are sometimes expanded by exon skipping during pre-mRNA processing. Skipping of certain exons during the splicing of mutant pre-mRNAs could generate in-frame 'deletions' in the final mRNA transcript, which restore the translational reading frame [25,28,29]. Moreover, mild BMD phenotypes, as well as transgenic *mdx* mice expressing internally deleted Becker-type cDNAs, show that such dystrophin molecules are functional. Interestingly, some have also suggested that a positive correlation exists between the amount of dystrophin detectable in muscle biopsies and the age at which DMD patients lose the ability to walk independently [24,30].

Extensive interest is now being shown in the use of antisense nucleic acids to modify gene expression, particularly in virus infections, including the first licensed antisense drug, Vitravene (CIBA Vision) [31], and in cancer (reviewed in Ref. [32]). Many more therapeutic regimes are currently in clinical trials [33]. However, in cases of inherited disease arising from loss-of-function mutations, a means of positive modulation of gene expression rather than mRNA destabilisation needs to be employed, which utilises many of the principles of antisense DNA-based therapeutics. One way in which this may be achieved is by the modulation of pre-mRNA splicing using 2′-*O*-methyl RNA antisense oligonucleotides (ASOs). The technique was pioneered by Kole et al. [34,35], who restored the correct splicing in a mutated β-globin gene with an extra exon leading to β-thalassaemia. It has since been used in a number of studies to induce skipping of a constitutively spliced exon in mouse dystrophin, and in a number of other systems [36–44]. Whilst most studies have focussed on the use of oligonucleotides containing 2′-*O*-methyl RNA modifications, others have pursued refinement of the technique by using splicomers based on 2′-methoxyethyl (MOE) modified sugar-phosphate backbones [40,42], and on oligo-peptide nucleic acids (PNA) [43].

2. Oligonucleotide array and functional screening for splicomer activity in mdx muscle cells

We have recently sought to improve the design of DMD gene splicomers using empirical approaches, focussing on exon 23 of the murine dystrophin gene, which carries a nonsense mutation in the *mdx* mouse model of DMD. If a reagent is active in modulating the splicing of the dystrophin pre-mRNA, the stop codon that is present in exon 23 is excluded from the translational reading frame, and expression of dystrophin is restored. We have now demonstrated the use of oligonucleotide array synthesis [45] to attempt to select optimally performing ASOs. This has shown that there is clearly a region that performs best in an in vitro hybridisation between a synthetic RNA and the array spanning the 5′ splice site of dystrophin exon 23. This is located very close to the splice site itself, suggesting that it may be a suitable target for modulation of dystrophin pre-mRNA splicing using ASOs. Previous studies using this type of scanning-array technology have shown a good correlation between the hybridisation of an ASO to a synthetic RNA and the ability of that ASO to direct RNase H cleavage of the pre-mRNA, both in vitro and in vivo [46–48]. In addition, this also correlates with the predicted secondary structure of the pre-mRNA. We were unable to show any substantial hybridisation between the synthetic pre-mRNA containing exon 23 and a scanning array of ASOs complementary to the 3′ splice site of exon 23. We have designed an overlapping set of 2′-*O*-methyl RNA ASOs which lie across the region defined by the scanning array hybridisation. All of these ASOs have been transfected into primary cultures of *mdx* mouse muscle, which do not express dystrophin, and their ability to induce the modulation of exon 23 splicing has been analysed, by RT-PCR of RNA isolated from the cells, 72 h after ASO treatment. This has demonstrated that some of the overlapping ASOs are more active than others in this assay, with the best being capable of skipping exon 23 in greater than 60% of dystrophin mRNA. A similar set of ASOs designed against the 3′ splice site of exon 23 were entirely ineffective in the cellular assay. We observed that the correlation between hybridisation efficiency in vitro, and the effectiveness of the ASOs in the assay in cultured muscle cells was not absolute. Whilst this partly contradicts previous work demonstrating the correlation of array hybridisation with in vivo activity [48], it seems to argue that there may be a mechanism at work in which the 5′ splice site itself is made more accessible to hybridisation by ASOs. One such mechanism may involve the putative ATP-dependent RNA helicase activity contained in a number of protein components of the spliceosome (reviewed in [49]). Whilst it is thought that these enable changes to take place in the formation of the higher order structures within the spliceosome, it is possible that at least part of their function is to ensure that the binding sites for U1, U2 and U5 snRNPs, all of which undergo base pairing with consensus sequences within the pre-mRNA, are held in an open conformation. This will in turn affect the secondary structure of the pre-mRNA, thereby offering an explanation for the differences between the in vitro hybridisation of the synthetic RNA to the array and the performance of the ASOs in cultured cells. However, it does not explain why the 3′ splice site of exon 23 was so poor a target for ASO action either in the in vitro hybridisation or in the cellular assay.

3. Splicomer-induced re-expression of dystrophin

In addition to the demonstration of high levels of exon skipping at the RNA level, we have also shown that treatment of cultured muscle cells with the most effective ASO is

capable of stimulating de novo production and localisation of dystrophin protein. However, this appears to occur at a relatively low level. The reason for such occurrence at this stage is unclear. We would postulate that the exclusion of exon 23 from the mature dystrophin mRNA leads to instability of either the mRNA or the protein, or perhaps both. It is known that the levels of dystrophin mRNA in *mdx* mouse muscle cells constitutes about 10% of the normal levels [50]. This is thought to be due to a scanning mechanism within the cell, whereby the presence of a stop codon within an open reading frame targets the mRNA for more rapid degradation. We would assume that the exclusion of exon 23, and hence the stop codon in question, would negate this targeted degradation, and lead to increased levels of dystrophin mRNA. Since our assay compares the full-length and the skipped mRNA directly, there is little evidence to suggest that there is a substantially increased level of dystrophin mRNA lacking exon 23. It is possible that the actual proportion of the total dystrophin pre-mRNA from which exon 23 is being deleted by the action of the ASOs is less than the 50% figure we quote here, but that the small amount of this skipped mRNA is more stable, and is accumulating during the period of the treatment. Our work compares favourably with that of Mann et al. [44], who recently reported the use of a different ASO that leads to the skipping of both exons 23 and 24, in approximately equal amounts. This was shown to lead to the accumulation of dystrophin protein following repeated intramuscular administration in mdx animals. Unfortunately, it is not yet possible, even by the use of exon specific antibodies, to distinguish between a protein that lacks exon 23 sequences, and one that lacks both exons 23 and 24. It is possible that Mann et al. have produced a protein of the latter variety, which may be more stable than that lacking only exon 23 sequences, and has thus accumulated. Whilst in this system, in which the exclusion of both exons 23 and 24 would be a frame restoring event, the exclusion of more than one exon would not be expected to have deleterious effects on the individual. The exclusion of more than one exon may not be desirable in other systems, or indeed the exclusion of other exons from human dystrophin, for example. In this respect, our superior empirical approach has allowed us to select an ASO which can specifically and uniquely exclude exon 23 in our model system.

The use of this class of reagent has been shown to be of considerable potential as an alternative use of genetic medicine. It has clear applications in the treatment of β-thalassaemias [34,35], cystic fibrosis [39], interleukin-5 receptor-mediated asthma [43,43], certain cancers [51] and possibly a range of other genetic and acquired diseases. In a large proportion of cases of DMD, one or more exons have been completely deleted. Splicomer-mediated removal of one or more further exons would be predicted to restore the reading frame, which could lead to an alleviation of the DMD phenotype, perhaps to a milder BMD-like disease state. The such study has been performed using exon 44, which is located in one of the hotspots for deletion in the human dystrophin gene. In this case [52], exon 45 has been skipped by the use of a 2′-*O*-methyl RNA oligonucleotide which is complementary not to a splice site, but to an exon recognition sequence (ERS) which lies within, and aids the selection of the exon. Whilst this observation is very encouraging for the potential use of ASOs to skip exon 45, not all exons contain an ERS. It seems likely, therefore, that the more generally applicable approach would be the targeting of splice sites. The success of the use of splicomers, as with more conventional gene therapy approaches for DMD, depends upon the treatment of a wide variety of muscles within an affected individual. This would certainly require systemic delivery, which is perhaps more feasible with ASOs than other reagents, but which still remains a barrier to the success of the approach.

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